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PCR-based detection of *Babesia bovis* and *Babesia bigemina* in their natural host *Boophilus microplus* and cattle

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Abstract

PCR and nested-PCR methods were used to assess the frequency of *Babesia bovis* and *Babesia bigemina* infection in *Boophilus microplus* engorged females and eggs and in cattle reared in an area with endemic babesiosis. Blood and the engorged female ticks were from 27 naturally infested calves and 25 crossbred cows. The frequency of both *Babesia* species was similar in calves and cows ($P>0.05$). *Babesia bovis* was detected in 23 (85.2%) calves and in 25 (100%) cows and *B. bigemina* was detected in 25 (92.6%) calves and in 21 (84%) cows. Mixed infections with the both *Babesia* species were identified in 42 animals, 21 in each age category. Of female ticks engorged on calves, 34.9% were negative and single species infection with *B. bigemina* (56.2%) was significantly more frequent ($P<0.01$) than with *B. bovis* (4.7%). Most of the females (60.8%) engorged on cows did not show *Babesia* spp. infection and the frequency of single *B. bovis* infection (17.6%) was similar ($P>0.05$) to the frequency of single *B. bigemina* infection (15.9%). Mixed *Babesia* infection was lower ($P<0.01$) than single species infection in female ticks engorged either in cows (5.7%) or in calves (4.3%). An egg sample from each female was analysed for the presence of *Babesia* species. Of the egg samples from female ticks infected with *B. bovis*, 26 (47.3%) were infected while from those from female ticks infected with *B. bigemina* 141 (76.6%) were infected ($P<0.01$). The results showed that although the frequency of both species of *Babesia* was similar in calves and cows, the infectivity of *B. bigemina* was higher to ticks fed on calves while to those ticks fed on cows the infectivity of both *Babesia* species was similar.

Keywords: *Babesia bovis*; *Babesia bigemina*; *Boophilus microplus*; Brazil; Polymerase chain reaction

1. Introduction

In Latin America, bovine babesiosis is due to *Babesia bovis* and *Babesia bigemina*, which are exclusively transmitted by the tick *Boophilus microplus*. Most of the Brazilian territory is endemic for *B. microplus*. While the condition of endemic stability is the most common for both *Babesia* species, areas of enzootic instability have been identified (Vidotto et al., 1997).

Information about the epidemiology of babesiosis, especially on the dynamics of transmission by the vector ticks is essential for the elaboration of adequate control strategies (Morzaria et al., 1992). Thus, in addition to information about the prevalence of these parasitic diseases in vertebrate hosts, the detection and specific discrimination of *Babesia* species in ticks is of fundamental importance.

Microscopic techniques for blood examination remain the most appropriate for the diagnosis of acute babesiosis, but the low sensitivity of these methods does not permit its use in epidemiological studies in which it is necessary to identify carrier animals (Almeria et al., 2001). Several serological methods standardized for the diagnosis of babesiosis have been extensively employed in epidemiological field studies. Among the drawbacks of these techniques is the occurrence of cross-reactions between *B. bovis* and *B. bigemina* (Passos et al., 1998) and the lack of discrimination between previous exposure and current infections (Wagner et al., 1992).

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The main drawbacks for the microscopic detection of *Babesia* spp. in haemolymph of adult ticks, and in tick egg and larval squashes are the low sensitivity and the difficulty of differentiating between the species involved (Guglielmone et al., 1996, 1997).

The application of PCR-based tests to the study of the epidemiology of babesiosis is still incipient, but characteristics of high sensitivity and specificity have been verified by several authors (Fahrimal et al., 1992; Figueroa et al., 1992; Smeenk et al., 2000; Almeria et al., 2001) for the detection of infection both in the vertebrate hosts and ticks.

Because low level infection as well as transmission and transmissibility are important aspects in epidemiology of babesiosis, PCR-based techniques were used in order to estimate the rate of *B. bovis* and *B. bigemina* infection in blood from cross-bred dairy cattle of two age ranges and in *B. microplus* females and eggs collected from these animals.

2. Materials and methods

2.1. Animals

The study was conducted on 52 crossbred (*Bos indicus*×*Bos taurus*) dairy animals (25 cows aged more than 3 years and 27 calves aged 1–4 months) naturally infested with *B. microplus*. All animals belonged to a herd reared at the 'Centro de Pesquisa de Pecuária do Sudeste-EMBRAPA', located in São Carlos, SP, Brazil (latitude 22° 01′ South and longitude 47° 53′ West), with a subtropical climate.

2.2. Blood samples and B. microplus collections

All animals were sampled only once and blood and *B. microplus* females were simultaneously collected on three occasions from November 2000 to January 2001. Blood samples from the jugular vein were collected into tubes containing EDTA for DNA extraction and samples from ear vessels were obtained for the preparation of thin blood smears, stained with Giemsa, for the determination of parasitemia.

All *B. microplus* females more than 4.5 mm (Hermans et al., 1994) were collected from each animal to determine the parasite burden. After the counts, 10 fully engorged females from each animal were individually placed on hollow polyethylene plates and incubated at 27 ± 1 °C and 85–86% relative humidity for collection of eggs.

On the fifteenth day of oviposition, each female tick was transferred to a labelled microtube, and each mass of eggs laid between the 6th and 15th day of oviposition (Mahoney and Mirre, 1977) was collected and transferred to another microtube. The female tick and egg samples were stored in a freezer at −80 °C for later DNA extraction.

2.3. DNA extraction and PCR and nPCR procedures

DNA was extracted from a blood sample aliquot of 300 μl using the GFX™ Genomic Blood DNA Purification kit (Amershan Bioscience) according to manufacturer's instructions.

For DNA extraction from the *B. microplus* females, each frozen specimen was individually macerated in a microtube to which 10 μl of buffer was added (10 mM Tris–HCl, 1 mM EDTA and 5% Triton X-100, pH 8.5) and incubated for 15 min at room temperature. In sequence, 20 μl of proteinase K solution (20 μg/ml in 10 mM Tris–HCl, pH 8.0) was added, the preparation was incubated in a water bath for 4 h at 56 °C, and for 10 min at 70 °C. After incubation, DNA extraction was performed using the GFX™ Genomic Blood DNA Purification kit according to the same protocol as used for the blood samples.

For DNA extraction from egg samples, 20 mg samples of eggs were weighed out and placed in a microtube and washed with buffer (10 mM Tris–HCl, 1 mM EDTA, 5% Triton X-100, pH 8.5). After centrifugation at 5000×*g* for 2 min, the supernatant was discarded and the eggs were macerated with the aid of a glass rod. A solution of Proteinase K (20 μl) was added to the egg macerate and the preparation was incubated overnight at 56 °C. After incubation, the same procedures as described for the female ticks were adopted.

PCR and nested-PCR (nPCR) techniques were used for the amplification of *B. bovis* and *B. bigemina* DNA using the primer sequences described by Figueroa et al. (1993). Only PCR-negative blood samples were submitted to nPCR, whereas all samples extracted from female ticks and eggs were submitted to nPCR.

PCR was performed in a 25 μl solution containing 10 mM Tris–HCl; 50 mM KCl; 1.5 mM $MgCl_2$; 1.5 U Taq-DNA-polymerase (Amersham Bioscience); 0.2 mM of each nucleotide (Amersham Bioscience); 10 pm of each primer and 5 μl of DNA samples. The same buffer at the same concentrations and 2 μl of the previously PCR amplified products were used for nPCR.

Purified samples of *B. bovis* and *B. bigemina*, kindly provided by Drs Raul H. Kessler and Cláudio Madruga (CNPGC-Embrapa, Brazil) were used for positive control of PCR and nPCR reactions. Amplification reactions using purified bovine DNA, and purified samples of *Anaplasma marginale* were performed to test the specificity of the primers. For the control of contamination, a tube containing no DNA sample was included in each reaction batch.

The amplification products were submitted to electrophoresis on 1.5% agarose gel containing ethidium bromide. The length of the amplified products was estimated by including a base pair standard (100 base-pair ladder - Amersham Biosciences) and the amplified products were visualized with an UV transilluminator. Samples presenting visible bands of approximately 278 base pairs (PCR) or 170 base pairs (nPCR) were considered positive for *B. bigemina*.

For *B. bovis*, PCR positive bands were of 350 base pairs and nPCR positive bands of 290 base pairs. Three *B. bigemina*-amplicons and three *B. bovis*-amplicons, respectively from blood, female tick and egg samples were sequenced and were confirmed to correspond to the GenBank accessions S45366 (ApeI - AvaI restriction fragment of *B. bigemina*) and AF030061 (rap-1 protein of *B. bovis*).

2.4. Estimated analytical sensitivity of PCR and nPCR

PCR and nPCR sensitivity was estimated from a 300 µl aliquot of the purified *B. bovis* and *B. bigemina* samples containing 3×10^6 and 6×10^6 parasitized erythrocytes, respectively. The samples were diluted 15 times by 10-fold dilutions and submitted to amplification. Sensitivity was calculated considering the number of parasitized erythrocytes detected in the last dilution in which it was possible to identify the bands of the corresponding size for each species.

2.5. Statistical analysis

The data obtained from calves and cows concerning blood DNA amplification (PCR and nPCR), and nPCR of eggs and female ticks were compared by the χ^2-test. The exact Fisher test was used to compare the data for the blood smears with the data of blood PCR and nPCR using the FREQ procedure of the SAS software (SAS, Institute Inc., SAS/STAT., 1996. User's Guide, version 6.11, 4ed., v.2, Cary, SAS Institute Inc.).

3. Results

3.1. Blood samples

Estimated analytical sensitivity of PCR and nPCR for *B. bigemina* corresponded to parasitemias of 0.00003 and 0.0000003%, respectively. For *B. bovis*, the sensitivity corresponded to parasitemias of 0.000017% (PCR) and 0.00000017% (nPCR).

The data concerning the detection of *B. bovis* and *B. bigemina* in blood samples using blood smears and DNA amplification are presented in Table 1. Merozoites of *B. bigemina* (0.1–0.2% parasitized erythrocytes) and *B. bovis* (less than 0.1%) were detected exclusively in the blood smears of calves, with only one of them carrying both species.

Table 1
Summary of detected *Babesia bovis* and *Babesia bigemina* merozoites (blood smears) and DNA (PCR/nPCR) in blood

	Calves ($n=27$)		Cows ($n=25$)	
	Blood smear	PCR(nPCR)	Blood smear	PCR(nPCR)
B. bovis	2	2(0)	0	2(2)
B. bigemina	11	3(1)	0	0(0)
B. bovis/*B. bigemina*	1	20(1)/14(7)	0	17(4)/14(7)

Table 2
Number of *Boophilus microplus* female infected with *Babesia bovis* and/or *Babesia bigemina* detected by nPCR in 258 ticks collected from 27 calves

Calf infection ($n=27$)	Female tick infection ($n=258$)			
	B. bovis	*B. bigemina*	*B. bovis*/ *B. bigemina*	Negative
B. bovis	0	10	1	5
B. bigemina	1	27	1	11
B. bovis/*B. bigemina*	11	108	9	74
Total	12	145	11	90

Twenty-two of the 23 calf blood samples positive for *B. bovis* were detected by PCR and one by nPCR, and of the 25 samples positive for *B. bigemina*, eight were detected by nPCR. Nineteen of the 25 cow blood samples positive for *B. bovis* were detected by PCR and six by nPCR, and 14 of the 21 samples positive for *B. bigemina* were detected by PCR. The rates of *B. bovis* and *B. bigemina* infection were similar for the two animal categories ($P>0.05$), and concomitant infection with the two *Babesia* species were detected in 21 cows and 21 calves.

3.2. Tick samples

The data concerning the detection of *B. bovis* and/or *B. bigemina* in *B. microplus* females according to infection of calves and cows are presented in Tables 2 and 3, respectively. For female ticks that engorged on calves (258), the number of *B. bigemina* infected females (145) was higher ($P<0.01$) than the number of females infected with *B. bovis* (12), the number of those with mixed infection with both species (11) as well the number of uninfected females (90). Of the 113 females that fed on the 12 calves with overt *B. bigemina* parasitemia, 79 (70%) were infected with this species, while of the 145 females that fed on calves without parasitemia, 77 (53%) were infected ($P<0.01$).

In the female ticks collected from cows, the frequency of *B. bovis* (43) and *B. bigemina* (39) infection were similar

Table 3
Number of *Boophilus microplus* female infected with *Babesia bovis* and/or *Babesia bigemina* detected by nPCR in 245 ticks collected from 25 cows

Cow infection ($n=25$)	Female tick infection ($n=245$)			
	B. bovis	*B. bigemina*	*B. bovis*/ *B. bigemina*	Negative
B. bovis	3	10	1	26
B. bigemina	–	–	–	–
B. bovis/ *B. bigemina*	40	29	13	123
Total	43	39	14	149

Table 4
Number of *Boophilus microplus* egg samples infected with *Babesia bovis* and/or *Babesia bigemina* as a function of the origin of the engorged females

Engorged female origin	Egg samples infection				
	B. bovis	*B. bigemina*	*B. bovis*/ *B. bigemina*	Negative	Total
Calves	10	126	5	86	227
Cows	16	15	0	180	211
Total	26	141	5	266	438

($P>0.05$), being higher ($P<0.01$) than the frequency of mixed infection (14) and lower ($P<0.01$) than the frequency of negative females (149).

The results of DNA amplification of *B. bovis* and *B. bigemina* in egg samples are presented in Table 4 according to the origin of the female ticks. For females that fed on calves, simple *B. bigemina* infection predominated in the egg samples (126) and for females that fed on cows non-infected egg samples (180) predominated.

The females collected from calves produced a significantly larger number ($P<0.01$) of egg samples infected with *B. bigemina* (131) than with *B. bovis* (15), whereas those collected from cows produced similar numbers of egg samples infected with each single species ($P>0.05$).

No significant association ($P>0.05$) was observed between the frequency of *B. bigemina* infection in *B. microplus* eggs and the presence of overt parasitemia in the vertebrate host. Of the 100 egg samples whose females engorged on calves with overt parasitemia, 64 (64%) were positive, and of the 127 egg samples originating from females engorged on calves without parasitemia 67 (52.75%) were positive ($P>0.05$).

Table 5 shows the number of *B. microplus* egg samples infected with *B. bovis* and/or *B. bigemina* according to infection of the females that gave origin to them, regardless of the origin of the female ticks. In this evaluation, the 431 data pairs analysed were those for which amplification results were obtained both for the females and their respective egg samples. A close correspondence between female tick results and their respective egg samples was observed only for *B. bigemina* infected and for negative female ticks. Most of egg samples (61.3%) from *B. bigemina* infected females (155) were also infected, and 77% of egg samples from negative females (210) were negative. For the females infected with *B. bovis* (45), 77.7% of the egg samples were negative, 4.4% were infected with this species, and no mixed infected egg samples were produced by mixed infected female ticks (21).

Table 5
Number of *Boophilus microplus* egg samples infected with *Babesia bovis* and/or *Babesia bigemina* in relation to the species of *Babesia* detected in the engorged females

Engorged female infection	Egg sample infection			
	B. bovis	*B. bigemina*	*B. bovis*/ *B. bigemina*	Negative
B. bovis	2	8	0	35
B. bigemina	8	95	3	49
B. bovis/ *B. bigemina*	0	7	0	14
Negative	16	32	0	162
Total	26	142	3	260

4. Discussion

This is the first study in which molecular diagnostic techniques were used to investigate the epidemiology of bovine babesiosis in Brazil and in which the rates of infection were assessed simultaneously in the cattle and in the intermediate host tick.

The high frequency of *B. bovis* and *B. bigemina* infections both in calves and in adult animals indicate a situation of stable endemy (Goff et al., 2002) and are comparable to those detected by immunodiagnosis in different endemic regions in the country (Araújo et al., 1997; Madruga et al., 2001). However, using PCR techniques the detection of specific infection was possible in animals as young as 1 month of age, and the prevalence obtained refers to the presence of current infection and not simply exposure to the protozoon, as is the case when serological methods are used (Wagner et al., 1992).

As expected, the frequency of *Babesia* infection in cattle detected by DNA amplification methods was significantly higher than that obtained in microscopic examination of blood smears, because the latter method does not detect positive animals in the early phase or in the carrier stage of infection, when the number of circulating parasites is very low (Calder et al., 1996).

The detection of *Babesia* infection in animals in the early phases of infection and in carrier animals by DNA amplification was a powerful tool for epidemiological investigation, since these animals represent an important source of alimentary infection of *B. microplus* females (Friedhoff and Smith, 1981), especially if we consider that vertical infection (=continuous transovarial and transtadial infection of all tick stages) does not occur in *B. bovis* and, although possible for *B. bigemina*, it is much less efficient (Büscher, 1988).

As the methodology used in the present study permitted the detection of current infections both in cattle and in ticks, and especially the discrimination between *Babesia* species, it was possible to quantitatively assess the contribution of young and adult animals to the induction of *B. microplus* infection by each *Babesia* species.

Babesia bigemina were detected more frequently in female ticks that fed on calves (156/258) than in those that fed on cows (53/245). In addition, calves with overt *B. bigemina* parasitemia were responsible for a larger number of infected female ticks than calves with negative Giemsa-stained smears. These results agree with previous reports about *B. bigemina* (Riek, 1964; Callow, 1968)

and with observations made on other *Babesia* species (Riek, 1966; Yeruham et al., 2001).

Several authors who investigated the quantitative aspects of *Babesia* transmission have reported that *B. microplus* females are more frequently infected with *B. bigemina* than with *B. bovis* (Riek, 1964, 1966; Mahoney and Mirre, 1971, 1977; Davey, 1981). However, it should be pointed out that these observations were made in experiments in which the ticks were fed on animals aged 4–8 months experimentally infected with one or the other *Babesia* species. So, the present results agree with these observations only with respect to the females that became infected on calves, in which the frequency of infection with *B. bigemina* (156/258) was higher than the frequency of infection with *B. bovis* (23/258). In contrast, in the females engorged on cows, the frequency of *B. bovis* infection (57/245) was similar to the frequency of *B. bigemina* infection (53/245).

With respect to *B. bovis*, the significantly lower number of female ticks that became infected on calves (23/258) compared to those that became infected on cows (57/245) may represent evidence that age-related effects interfere in different ways with the infectivity of the two *Babesia* species for ticks. If in relation to *B. bigemina*, the higher rate of infection in the female ticks collected from young animals could be related to a greater parasite density in their blood and the lack of active immunity, the same does not apply to *B. bovis* for which the number of infected females was smaller for those that fed on young animals. Although there are no data to explain these results, the presence of foetal haemoglobin (HbF) in the calves could represent a possibility since HbF is considered one of the factors contributing to the high resistance of young cattle against *B. bovis* infection (Ristic and Levi, 1981). The presence of HbF, as well as some human haemoglobinopathies (Pasvol et al., 1976), is also related to greater resistance to infection with *Plasmodium falciparum*. Furthermore, significant differences were observed in the rate of infection of *Anopheles* mosquitoes feeding on individuals with different haemoglobin phenotypes (Robert et al., 1996). In addition to this circumstantial evidence suggesting that HbF may interfere with the transmission of *B. bovis* from calves to the female ticks, it should be pointed out that one of the mechanisms by which the ticks defend themselves from microorganisms is related to the peptides derived from enzymatic cleavage of the haemoglobin ingested by the ticks (Fogaça et al., 1999).

It is interesting to note that concomitant infection with the two *Babesia* species prevailed in the cattle of both age ranges, in contrast to what is observed for *B. microplus* females and eggs in which prevailed simple babesial infection. Recently, Paul et al. (2002) detected the existence of interspecific competition between two avian *Plasmodium* species and observed that this phenomenon occurs during the phase of gamete fertilization still inside the erythrocytes ingested by the mosquitoes. As also observed here, these authors verified that apparently one species does not interfere with the other in vertebrate hosts. Although it may be suspected that some type of competition occurs between *Babesia* species in a *B. microplus* tick, future investigations are necessary to confirm this possibility.

According to available epidemiological data, the percentage of female ticks that transmit *Babesia* spp. to their progeny is low even when they become infected on animals in the acute phase of the disease (Mahoney and Mirre, 1971). In the present study, since DNA was amplified using 20 mg aliquots of eggs, the data presented refer to the percentage of infected egg samples. On this basis, it can be seen that the frequency of egg samples infected with *B. bigemina* (33.3%) was significantly higher than that observed for *B. bovis* (7.1%), in agreement with previous observations.

There was no significant difference between the percentages of egg samples infected with *B. bigemina* in samples collected from calves with overt parasitemia (64.6%–64/99) and those from calves with no parasitemia (52.8%–67/127). A positive correlation between *B. bigemina* parasitemia in the vertebrate host and the rate of larval infection via feeding adult female ticks was reported by Riek (1964) but was questioned by others (Mahoney and Mirre, 1971; Melendez and Forlano, 1996). The present results show that, with respect to *B. bigemina*, there was a weak correlation between parasitemia and infection of the tick progeny, and that female ticks engorged on animals with undetectable parasitemia, but PCR-positive, transmit the infection to eggs at relatively high levels.

Despite the high sensitivity and specificity of PCR/nPCR, the occurrence of false-negative results has been recorded and has been attributed mainly to the presence of polymerase-inhibiting substances in the samples analysed (Hayden et al., 1991; Barker et al., 1994). In the present experiment, although some data could suggest the occurrence of false-negative results in the vertebrate hosts, these results should be interpreted with caution. It was observed that 22 *B. microplus* females infected with *B. bigemina* had engorged themselves on animals infected only with *B. bovis* (Tables 2 and 3), while only one of the females infected with *B. bovis* originated from an animal infected only with *B. bigemina*. In view of the possibility of vertical transmission exclusively for *B. bigemina* (Büscher, 1988), it is possible that the percentage of truly false-negative results in vertebrate hosts is lower than that suggested by the data obtained in the present study. On this basis, truly false-negative results seem to be related to the sensitivity of the method more than to the presence of inhibitory substances.

Further possible occurrence of false-negative results is observed when the infection of *B. microplus* egg samples is analysed in comparison with female tick infection. It was verified that eight egg samples infected with

B. bigemina were from females in which only *B. bovis* was detected. Similarly, eight egg samples infected with *B. bovis* originated from female ticks infected with *B. bigemina*. Besides these, negative females gave origin to 16 egg samples infected with *B. bovis* and to 32 infected with *B. bigemina*. In all these cases it is also possible that the false-negative female tick results are related to the detection threshold of the methods, since sporokinetes perform several cycles of sporogony in the eggs, increasing the availability of DNA strands for amplification.

In the present study, DNA amplification techniques verified that in an endemic area the infection rates of the two *Babesia* species is similar in adult and young cattle, but the transmissibility of each species for ticks varies as a function of the age of the vertebrate hosts.

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